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14. ABSTRACT

Fragile X Syndrome (FXS) is a single gene disorder caused by loss of *FMR1* gene function. This disease leads to cognitive impairment and is the most common genetic cause of autism, accounting for 2-6% of all diagnosed cases (Hagerman et al 2008). In previous studies of a *Drosophila* model for FXS, we identified pharmacological treatments that rescued phenotypes relevant to this syndrome such as social, neuroanatomical and cognitive deficits (McBride et al., 2005; Choi et al., 2010). These results have been translated to the mouse model of FXS leading to the impetus to initiate clinical trials with Fragile X patients (Yan et al., 2005; Dolen et al., 2007; de Vrij et al., 2008; Choi et al., 2011). The fact that clinical trials of two distinct compounds identified in flies and tested in mice have reported some level of efficacy highlights the relevance of *Drosophila* and mouse-based disease modeling to identify potential treatments for developmental brain disorders and other diseases (Berry-Kravis et al., 2008; Berry-Kravis et al., 2009;

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Introduction

Fragile X syndrome is the leading cause of intellectual disability resulting from a single gene mutation. Previously, we characterized social and cognitive impairments in a *Drosophila* model of Fragile X syndrome and demonstrated that these impairments were rescued by treatment with metabotropic glutamate receptor (mGluR) antagonists or lithium. In the mouse model of Fragile X a well-characterized phenotype is enhanced mGluR-dependent long-term depression (LTD) at Schaffer collateral to CA1 pyramidal synapses of the hippocampus. Herein, we have now identified a novel drug target in the mGluR signaling pathway, phosphodiesterase-4 (PDE-4), and demonstrate PDE-4 inhibition as a therapeutic strategy to ameliorate memory impairments in the *Drosophila* model of Fragile X. Furthermore, we examine the effects of PDE-4 inhibition by pharmacologic treatment in the Fragile X mouse model. Acute inhibition of PDE-4 by pharmacologic treatment in hippocampal slices rescues the enhanced mGluR-dependent LTD phenotype. Additionally, chronic treatment of Fragile X mice in adulthood with a PDE-4 inhibitor for eight weeks also restores the level of mGluR-dependent LTD to those observed in wild type (WT) animals. Translating the findings of successful pharmacologic intervention from the *Drosophila* model into the mouse model of Fragile X syndrome is an important advance, in that this identifies and validates PDE-4 inhibition as potential therapeutic intervention for the treatment of individuals afflicted with Fragile X syndrome.

A) Completion of PDE4 studies in the *Drosophila* fragile X model. (items 2a, 4a and b, 5a, and 8a on Statement of Work.)

For Task 2a we have established several stocks to genetically validate the results that indicate that pharmacological inhibition of Gsk3beta activity can rescue Short-term memory, as well as to examine the impact of targeting downstream members of the mGluR signaling pathway. These stocks are currently being validated.

Task 4: In studies to examine the effects of PI3K antagonists on the *dfmr1* mutants we have found that the naïve courtship and short-term memory phenotypes can be rescued. These results have been validated genetically as shown below. Interestingly we have also been able to rescue the circadian defect displayed by the *dfmr1* mutants. The circadian phenotype however was not rescued by the pharmacological treatment. In experiments unrelated to this grant we have found that the critical period for decreasing PI3K activity with respect to circadian behavior is during the pupal period, a time in development that we cannot administer pharmacological agents, easily.

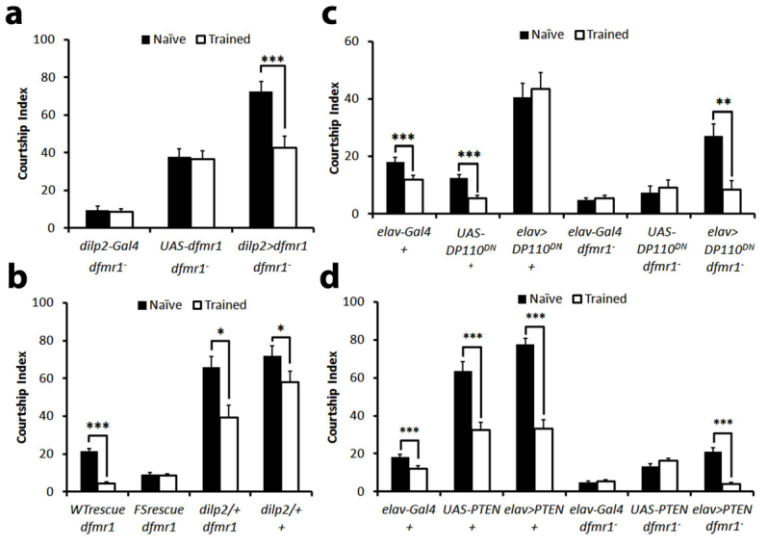


Figure 1| The short-term memory defect of *dfmr1* mutant flies is rescued by expression of *dfmr1* in the IPCs, genetic reduction of PI3K signaling activity. Graphs depict courtship indices of naïve males (black) and males trained with a mated female (white). **a,** Expression of *dfmr1* in the IPCs of *dfmr1* mutant flies restores memory, $p<0.001$. Memory is not seen in *dfmr1* mutants carrying the *dilp2-Gal4* or *UAS-dfmr1* transgenes alone. **b,** STM is restored in *dfmr1* mutants heterozygous for a *dilp2* mutation, $p<0.05$. **c,** STM is restored in *dfmr1* mutants expressing $DP110^{DN}$ pan-neuronally, $P<0.01$. **d,** STM is restored in *dfmr1* mutants over-expressing PTEN pan-neuronally, $P<0.001$. All error bars depict s.e.m.

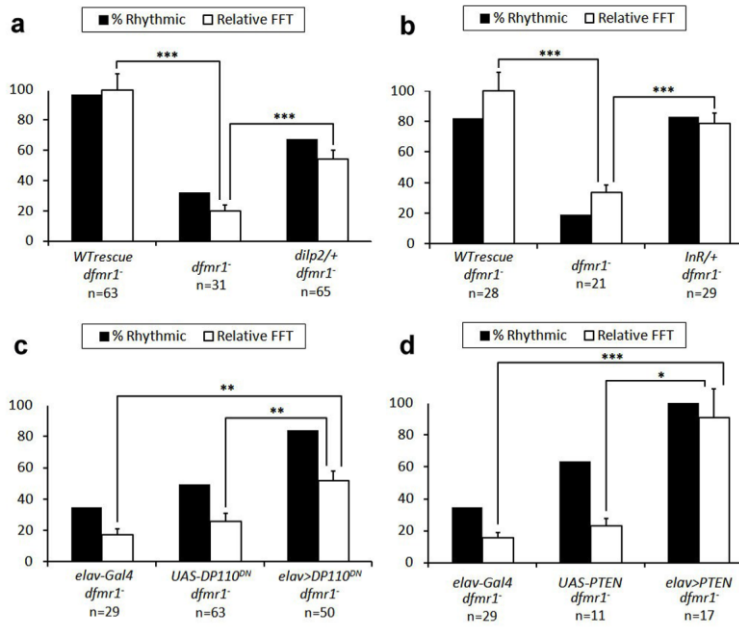


Figure 2| Genetic reduction of the insulin pathway rescues the circadian defect observed in *dfmr1* mutants. a-d, Panels show the percent rhythmic (black) and relative FFT values (white) for genetic combinations testing the effect of reducing insulin signaling in *dfmr1* mutants on circadian behavior. **a,** Circadian behavior of *dfmr1* mutants with the *WTrescue* transgene or with one copy of a null allele of *dilp2* (*dilp2*/+, *dfmr1*$^{-}$) display significantly improved circadian behavior relative to *dfmr1* mutants, p<0.001. **b,** Circadian behavior of *dfmr1* mutants with the *WTrescue* transgene or with one copy of a mutant allele of the insulin receptor (*InR*/+, *dfmr1*$^{-}$) display significantly improved circadian rhythmic strength relative to *dfmr1* mutants, p<0.001. **c,** Circadian behavior of *dfmr1* mutants with both *elav-Gal4* and *UAS-DP110*^{DN} (*elav>DP110*^{DN}, *dfmr1*$^{-}$) display significantly improved rhythmicity relative to *dfmr1* mutants with either transgene alone (*elav-Gal4, dfmr1*$^{-}$) and (*UAS-DP110*^{DN}, *dfmr1*$^{-}$), p<0.01. **d,** Circadian behavior of *dfmr1* mutants with both *elav-Gal4* and *UAS-PTEN* (*elav>PTEN, dfmr1*$^{-}$) display significantly improved circadian behavior relative to *dfmr1* mutants with either transgene alone (*elav-Gal4, dfmr1*$^{-}$) and (*UAS-PTEN, dfmr1*), p<0.001 and p<0.05 respectively. Significance denoted as described in Fig. 1. Error bars represent s.e.m.

Task 5: We have tested the effect of treating the *dfmr1* mutants with the Gsk-3beta inhibitor AR-A014418 during development and in adults. While we have not detected an effect on naïve courtship, we have found that treatment of *dfmr1* mutants during adulthood rescues the short-term memory deficit.

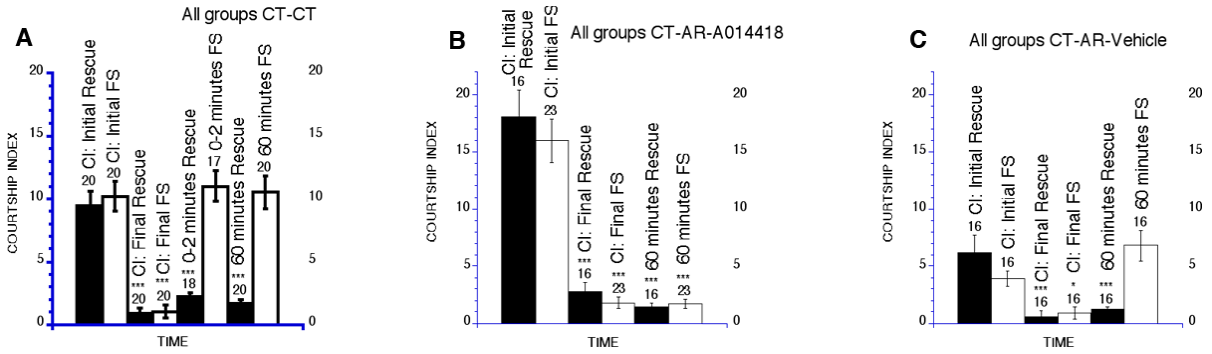


Figure 3. Treatment of the *dfmr1* mutants with the Gsk 3beta inhibitor AR-A014418 rescues short term memory. A) Both *dfmr1*mutants (FS) and controls (Rescue) display normal learning during training (Intial vs Final). The mutants however fail to display memory (reduced courtship) after the training. B) Treatment with AR-A014418 during adulthood rescues the short-term memory deficit (60 minutes) and has no negative effect on memory in the controls. C) Vehicle treatment for AR-A014418 has no effect on memory.

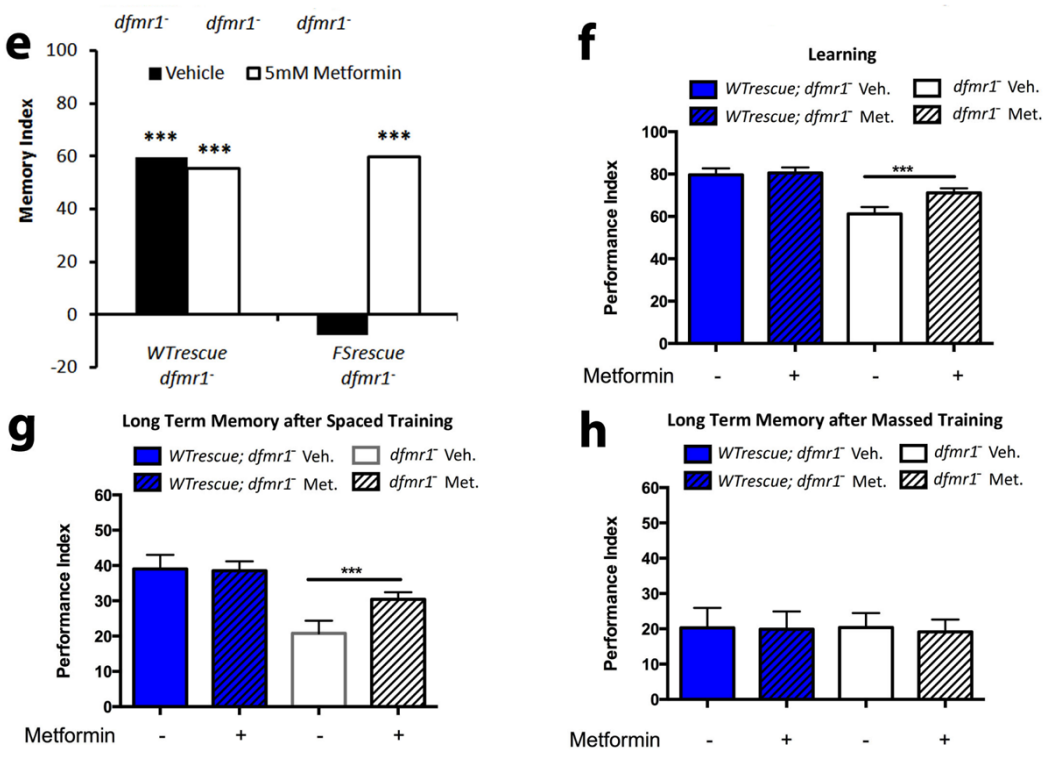
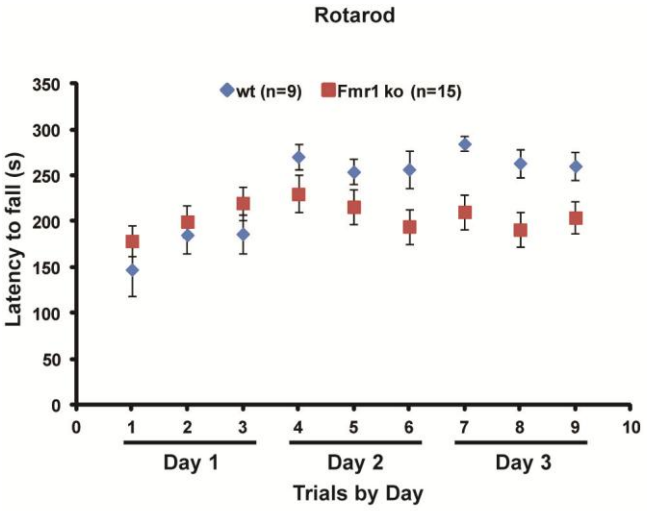
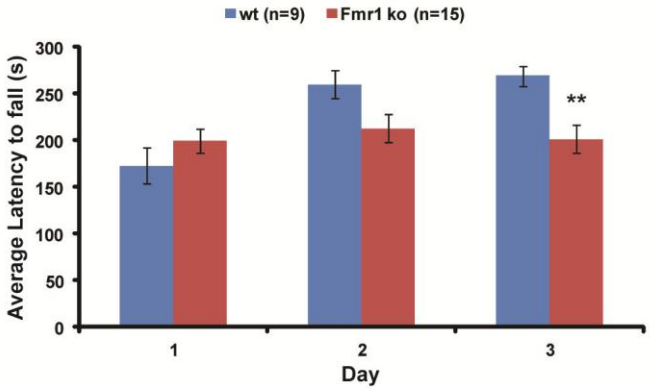


Figure 4| Treatment with metformin rescues memory in *dfmr1* mutants. Panels show the effect of metformin treatment on memory. **e,** STM is measured using memory index (MI) calculated from the courtship indices (CIs) of naïve (untrained) and trained flies using the formula $MI=(CI_{naive}-CI_{trained})/CI_{naive}$. Error bars are not shown for memory indices. **e,** *dfmr1* mutants treated with metformin demonstrated STM, $p<.001$ in contrast to *dfmr1* mutants treated with vehicle alone which did not demonstrate memory. N ranges from 36-86. **f-h,** Flies were also tested using the classical olfactory conditioning memory paradigm. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. **f,** Immediate olfactory conditioning memory, named learning, was significantly improved in *dfmr1* mutants after being administered a dose of metformin ($p <0.0001$).No effect was observed in wild-type controls. N = 6 PIs per group. **g,** One day memory after spaced training is significantly improved in *dfmr1* mutant flies after being given a dose of metformin overnight before training. ($p <0.00018$) N = 8 PIs per group. **h,** One day memory after massed training did not differ between groups. N = 8 PIs per group. Graph depicts mean ± s.e.m.

Task 12c. In testing the *dfmr1* mutants for behavioral phenotypes, we have found that few reported phenotypes could be reproduced in our hands using the Bl6 genetic background. We have therefore initiated testing in a 129XBl6 background and so far have found phenotypes to be more clear. We have now established conditions to perform the rotorod test that show a clear and reproducible difference between the mutants and littermate controls. We also have initial results that indicate the that object displacement assay is revealing a phenotype and the Y-maze is displaying a phenotype, however more n's need to be tested in these last two assays to determine if we can obtain statistically significant differences (not shown).


Rotarod
wt (n=9)
Fmr1 ko (n=15)
Latency to fall (s)
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
Day 1
Day 2
Day 3
Trials by Day


wt (n=9)
Fmr1 ko (n=15)
Average Latency to fall (s)
300
250
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100
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Day

Reportable outcomes:
Key Research Accomplishments:

Task 2a-The outcrossing of *Gsk-3beta*, *IPPase*, *InsP3R*, *Rheb*, *S6K* mutant stocks and the transgenic stocks *UAS-AMPK*, *UAS-4EBP* is complete we are now in the process of initiating the behavioral testing with these stocks.

Task 4. Test PI3K antagonists on the *Drosophila* fragile X model.
4a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone.
4b. Genetically validate the results obtained with the PI3K inhibitors.

Task 5. Test Gsk-3Beta antagonists on the *Drosophila* fragile X model.
5a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone.

8a. Examine naïve courtship, learning during training and memory in *dfmr1* mutants and controls treated with metformin vehicle during development alone, adulthood alone and during both times.

Ongoing tasks:

Task 1c. Perform biochemical analysis to determine effects of PDE-4 inhibition on PI3K and Akt activity and smRP6 levels.

Using an elisa assay to quantitate cAMP levels, we have now established that the *dfmr1* mutants have reduced resting levels of cAMP. We have also determined that treatment with rolipram can rescue the deficit of cAMP. Therefore we are in a position to now examine the effect of PDE-4 inhibition on PI3K, Akt and smRP6 levels.

2b. Molecular and genetic validation of genetic stocks. The *$InsP_3R$*, *dfmr1* and *Rheb*, *dfmr1* stocks may take additional time as the genes are genetically close to *dfmr1* on the 3rd chromosome.

5b. Genetically validate the results obtained with the Gsk-3Beta inhibitors.
5c. Perform biochemical analysis to determine effects of Gsk-3Beta inhibition on PI3K and Akt activity and smRP6 levels.

8a. Examine naïve courtship, learning during training and memory in *dfmr1* mutants and controls treated with AlCAR and vehicle during development alone, adulthood alone and during both times.

12c. Perform behavioral testing battery on *FMR1* KO and control mice.

13c. Perform behavioral testing on *FMR1* KO and control mice that are treated with PDE- 4, PDE-8 inhibitors or vehicle.

Manuscripts Accepted:
Wolman MA, de Groh ED, McBride SM, Jongens TA, Granato M, and Epstein JA. Modulation of cAMP and ras signaling pathways improves distinct behavioral deficits in a zebrafish model of neurofibromatosis type 1. Cell Rep. 2014 Sep 11;8(5):1265-70. doi: 10.1016/j.celrep.2014.07.054. Epub 2014 Aug 28.

Manuscripts under review:

PDE-4 inhibition rescues aberrant synaptic plasticity in Drosophila and mouse models of Fragile X syndrome. Choi C.H., Schoenfeld B.P., Bell A.J., Hinchey J., Choi R.J., Hinchey P., Kollaros M., Gertner M.J., Ferrick N.J., Terlizzi A.M., Yang Y., Woo N.H., Tranfaglia M.R., Siegel S.J., McDonald T.V., **Jongens T.A.**, McBride S.M.J.. J. of Neuroscience (resubmitted).

Papers under revision:

Insulin Misregulation underlies Behavioral and Cognitive Deficits in a *Drosophila* Fragile X Model
Monyak, R., Emerson, D, Zheng, X., Schoenfeld, B., McBride, S.M.J, Sehgal, A., and Jongens, T.A.

Conclusions:

The overall objective of the work we have accomplished so far was to examine the efficacy of pharmacologically inhibiting PDE-4 activity to correct synaptic plasticity impairments in the fly and mouse models of Fragile X syndrome. The *Drosophila* Fragile X model recapitulates the most debilitating aspect of the disease in humans, namely impaired cognitive function. In our further dissection of the proteins involved in the mGluR signaling cascade, we identified PDE-4 as a potential substrate whose inhibition may be beneficial in restoring proper intracellular signaling in the Fragile X model (Fig. 1A). Based on the fly data, tissue culture work, the mouse model and samples from humans afflicted with Fragile X syndrome, we speculated that cAMP levels are suppressed (Berry-Kravis and Sklena, 1993; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998; McBride et al., 2005; Kelley et al., 2007). PDE-4 inhibition should increase cAMP signaling by preventing the breakdown of cAMP that is produced during synaptic stimulation. Fragile X flies chronically treated in adulthood with PDE-4 inhibitors, or with genetically reduced levels of PDE-4, demonstrated intact immediate recall and short-term memory, validating PDE-4 inhibition as a potential novel therapeutic target for the treatment of synaptic plasticity impairments in Fragile X. We have validated the findings that indicate that cAMP levels are decreased in *dfmr1* mutants and that they are normalized by treatment with the drug rolipram. This finding adds to the growing body of literature demonstrating that pharmacologic treatment initiated in adulthood may have efficacy for the treatment of cognitive disorders that are already present in childhood as was first demonstrated in animal models of Fragile X and Neurofibromatosis type 1 in 2005 (Li et al., 2005; McBride et al., 2005; for review see Raymond and Tarpey, 2006; or Walsh et al., 2008).

In summary or work demonstrates that PDE-4 inhibition is a novel therapeutic target for the treatment of Fragile X. Prior to this work, it has only recently been demonstrated that enhanced LTD in the Fragile X model could be abrogated by chronic pharmacologic treatment (Choi et al., 2011). Equally as important is the demonstration that treatment in adulthood alone can rescue the phenotype, meaning that the phenotype is not irreversibly determined by pathogenic developmental circuitry. These findings urge the need for further exploration of PDE-4 inhibition as a potential therapy in Fragile X patients and in animal models of fragile X. Additionally, this work is a stepping stone for the field to begin a further pharmacologic dissection of the pathogenic signaling leading to aberrant LTD in the Fragile X model mouse, with the hope of these findings allowing the treatment of patients afflicted with Fragile X.

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